DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Industrial Application]This invention relates to an undifferentiated cell lump which produces the manufacturing method of saponin, and saponin, and a manufacturing method for the same.
[0002]
[Description of the Prior Art]The glycoside widely distributed over the plant kingdom, such as saponin, is little as an ingredient contained in vegetation.
When you needed them for the large quantity, it needed a lot of vegetation.
Therefore, it is extracted by the mass culture of the plant cell in recent years.
[0003]For example, in the saponin synthetic method in a medicinal ginseng radix cultured cell. the cultured cell in which the whole specialized morphologically is used for the cultured cell used as a component extraction raw material in part -- **** (Furuya, T., et al., Planta medica, 48, 83 (1983)). The saponin obtained from these cultured cells had much saponin with many same **** as saponin of plant body content, and, moreover, the propagation activity of those cultured cells was low. Although the medicinal value which is not in conventional saponin was expected, the composition needed to hydrolyze chemically or enzymatically the sugar chain of saponin with many usual ****, and saponin with few **** needed to perform re-****** of the aglycon obtained, and was not obtained easily.
[0004]
[Problem(s) to be Solved by the Invention]This invention provides the cell which produces the manufacturing method of saponins and these saponins for obtaining the short sugar chain saponin it is expected that the medicinal value which is not in conventional saponin is from a medicinal ginseng radix plant body.
[0005]
[Means for Solving the Problem]As a result of repeating research in view of the above-mentioned art, this invention person makes it pass through a dedifferentiation state beyond fixed time, and ranks second a callus derived from a medicinal ginseng radix plant body, By cultivating on redifferentiation conditions and removing a cell lump which redifferentiates, high fecundity was able to be shown, and a uniform undifferentiated cell lump's cultured cell system was able to be established, and saponin which this undifferentiated cultured cell produces was able to be obtained.
[0006]This invention an organization of a medicinal ginseng radix plant body by a culture medium for callus induction Namely, after subculture, It is a manufacturing method of saponins characterized by cultivating an undifferentiated cell lump obtained by removing a cell lump which does subculture and redifferentiates an obtained callus by a culture medium for redifferentiation derivation at the time of this subculture, and performing component extraction.
[0007]This invention cultivates a callus which carried out subculture of the callus derived from a medicinal ginseng radix plant body by a culture medium for callus induction, and elasticity-ized it by a culture medium for redifferentiation derivation, and is a manufacturing method of an undifferentiated cell lump removing a cell lump which redifferentiated.
[0008]This invention is also an undifferentiated cell lump which does not redifferentiate by a culture medium for redifferentiation derivation and which is derived from a medicinal ginseng radix plant body.
[0009]In this invention, a medicinal ginseng radix plant body is the vegetation of an araliaceous

(Araliaceae) Panax group, For example, Panax schinseng (Panax ginseng C.A.Meyer), Panax japonicus (Panax japonicus C.A.Meyer), etc. are mentioned, and especially Panax japonicus is preferred.

[0010]A thing containing a phytohormone which could use a culture medium generally used for culture of a plant cell, for example, added a phytohormone to MS basal medium or B5 basal medium can be used for a culture medium for callus induction used for this invention.

[0011]The above-mentioned culture medium usually A nitrate, ammonium salt, phosphate, sulfate, K, 0.5 to 1% of agar is added including organic substances, such as autotrophy salts, such as Fe and Mg salt, vitamins, amino acid (nicotinic acid etc.) (glycine etc.), and a myo inositol, and 1 to 5% of carbon sources (sucrose, glucose, fructose, malt sugar, etc.), and it is good also as a solid medium.

[0012]It is preferred for auxin and cytokinin to be mentioned and to use auxin and cytokinin together as a phytohormone added to the above-mentioned culture medium.

[0013]As the above-mentioned auxin, natural auxin (Indole-3-acetic acid, Indole-3-acetonitrile, etc.), Synthetic auxins (2,4-Dichlorophenoxyacetic acid, Indole-3-butyric acid, etc.) are mentioned, and gibberellin is removed. If it is the concentration, it is good to use 2 and 4-Dichlorophenoxyacetic acid, and it can derive a callus efficiently.

[0014]It is preferred for natural cytokinin (Zeatin etc.) and synthetic cytokinin (Kinetin, 6-Benzylamino purine, etc.) to be mentioned, and to use 6-Benzylamino purine as the above-mentioned cytokinin.

[0015]The above-mentioned phytohormone makes auxin indispensable and more than a kind should just contain it at least.

[0016]addition concentration of the above-mentioned phytohormone -- 10^{-7}-10^{-5}M -- it is 10^{-6}-10^{-5}M preferably.

[0017]When using auxin and cytokinin together, concentration of cytokinin is 10^{-7}-10^{-5}M and it is preferred that auxin concentration is more than concentration of cytokinin.

[0018]a culture medium for redifferentiation derivation of this invention changes auxin of the above-mentioned culture medium for callus induction to natural auxin except JIBERERINN, or its derivative -- the concentration -- 10^{-7}-10^{-5}M -- it is 10^{-7}-10^{-6}M preferably. As for addition concentration of cytokinin, it is preferred to be referred to as 1 / 10 - 1/100 of addition auxin.

[0019]Natural auxin except above-mentioned JIBERERINN, or its derivative, Specifically Indole-3-acetic acid and Indole-3-butyric acid, Indole-3-acetonitirile and Indole-3-propionic acid, 4-Chloroindole-3-acetic acid, Indole-1-acetic acid, 2-Methyl-indole-3-acetic acid and alpha-Naphthaleneacetic acid are mentioned, and it is Indole-3-butyric acid preferably.

[0020]Its Kinetin is preferred although cytokinin can use the same thing as a culture medium for callus induction.

[0021]Although a medicinal ginseng radix plant body is first organized for subculture by a culture medium for callus induction in this invention, 3-10 generations of passage cycles are every four to five weeks preferably in every four to six weeks, and the number of these subculture is usually 3-7.

[0022]A callus which passed three or more generations serves as elasticity as compared with a callus in early stages of derivation.

[0023]The passage of this elastic callus is carried out to a liquid medium excluding agar from a culture medium for callus induction, and 18-27 ** of dark places in 20-25 ** and 100-120-rpm rotary shaking culture are performed preferably.

[0024]A callus is increased briskly [0.3-2 cm in diameter a capitulum lump's gestalt] by this fluid shaking culture.

[0025]Next, although subculture is performed for an obtained capitulum lump by a culture medium for redifferentiation derivation, in every two to five weeks, one or more generations, this subculture period is every two to three weeks, and is two or more generations preferably.
[0026]Although there is a cell lump which redifferentiates at the time of culture in culture by the above-mentioned culture medium for redifferentiation derivation, such a cell lump removes and cultivates only an undifferentiated cell lump which does not redifferentiate.
[0027]As a method of removing a cell lump which redifferentiates, it may pick up with tweezers, a metallic needle, etc., or a mesh which has a 0.5-2-cm hole may be used.
[0028]Passage maintenance of the undifferentiated cell lump obtained by several judgment removal is carried out as a uniform cultured cell system, without redifferentiating.
[0029]18-27 ** is 20-25 ** preferably about the above-mentioned undifferentiated cell lump, and 100-120-rpm rotary shaking culture is performed.
[0030]Publicly known methods, such as a column and HPLC, are followed as a method of extracting saponin from the above-mentioned undifferentiated cell lump or its culture medium.
[0031]When a manufacturing method of this invention is performed by Panax japonicus, two kinds of saponin is obtained, and one is a compound of structure known, but another is a compound which is not obtained from Panax japonicus.
[0032]In a compound expressed, glucose, galactose, and mannose are illustrated as 1-hexose by the general formula (1), (2), and (3) obtained by a manufacturing method of this invention.
[0033]In a compound expressed with the general formula (2) and (3), as a combination of R_1 and R_2, R_1 and R_2 -- hydrogen and R_1 -- with hexose, hydrogen and R_1 are mentioned for R_2, three kinds of hexose ** are mentioned for R_2 from hydrogen, and R_1 and R_2 are hydrogen preferably.

[0034]Although a culture medium for redifferentiation derivation performs an undifferentiated cell lump's mass culture in this invention, a microorganism or its extraction ingredients, such as bacteria which raise each substance on a carbon source and acetic acid mevalonate pathway, plant sterol biosynthetic inhibitor, and saponin production to a culture medium for redifferentiation derivation, -- it can be independent, or it can combine, can add and mass culture can also be carried out.
[0035]
[Effect of the Invention]According to this invention, by obtaining the cultured cell system which has high propagation activity from a medicinal ginseng radix plant body by the above-mentioned tissue culture method, and produces saponin, saponin can be supplied adequately through every year and it can use as foodstuffs, a crude drug, or a medicine manufacture raw material of a medicine field.
[0036]
[Example]Hereafter, an example explains this invention concretely.
[0037]The spontaneous Panax japonicus (Panax japonicus C.A.Meyer) plant body was collected for an example 1 Kyoto forest in the prefecture. Surface sterilization of the rhizome of a plant body is carried out 70% in the order of MeOH, 1% hypochlorous acid, and 3% hydrogen peroxide solution, About the meristematic tissue portion of a rhizome, it is Murashige and Skoog in 1962. Nutrient salt of (MS) (Table 1), 2%Sucrose, 10 $^{-6}$M 2, 4-D (2, 4-Dichlorophenoxyacetic acid), It planted in the culture medium for callus induction of the falcon petri dish of a presentation of 10 $^{-6}$M BAP (6-Benzyl-amino purine) and 0.7% agar. When cultivated in a 25 ** dark place, the protean cell lump (callus) arose from the explant about two weeks afterward.

[0038]Only the produced callus was transplanted to the same fresh culture medium about one month afterward, and subculture was performed every four weeks after that. A callus will be the 3-4th generation from callus induction with white elasticity, and it came to increase briskly.
[0039]From derivation, to the 6th generation, the callus was transplanted to the liquid medium of the 100-ml flask (the amount of culture media: 40 ml) of the presentation excluding agar from the culture medium for callus induction, and 23 **, a dark place, and 120-rpm fluid rotary shaking culture were started. The incubator was scaled up 20 days afterward in a 500-ml flask (the amount of culture media: 120 ml), and subculture was carried out to day by day [20] after that. This fluid shaking culture cell was increased with 3-20 mm in diameter a capitulum lump's gestalt.
[0040]MS nutrient salt start fluid shaking culture and excluding NH_4 voice nitrogen (NH_4NO_3) in the capitulum lump of the 3rd generation (NH_4freeMS), When replanting in the liquid medium (culture medium for redifferentiation derivation) of Sucrose, 10^{-6}M IBA (3-Indole butyric acid), and 10^{-7}M Kinetin 2%, in the capitulum lump, the cell lump which the adventitious root produced was checked by the 2-3rd generation of the passage. As a result of repeating separation with the cell lump by which generating of a capitulum lump and an adventitious root is seen for every subculture, two kinds of cultured cell systems, a capitulum lump system and an adventitious root system, were obtained.
[0041]The adventitious root system was the liquid medium (culture medium for fluid shaking passages) which changed the nutrient salt of the culture medium for redifferentiation derivation into the nutrient salt (Table 1) of B5 culture medium in 1975, the root was elongated good and branching of the root was also observed.
[0042]Similarly, the capitulum lump also grew well by the culture medium for fluid shaking passages, and showed about 40-g fresh weight increment (about 16 times) to the amount of thickets of 2.5 g at the time of a passage by culture for 20 days.
[0043]
[Table 1]

MS培地とB5培地の栄養塩の成分表

成分	MS栄養塩 (mg/l)	B5栄養塩 (mg/l)
KNO_3	1900	2500
NH_4NO_3	1650	
$(NH_4)_2SO_4$		134
$MgSO_4 \cdot 7H_2O$	370	250
$CaCl_2 \cdot 2H_2O$	440	
KH_2PO_4	170	
$NaH_2PO_4 \cdot H_2O$		150
$MnSO_4 \cdot H_2O$		10
$MnSO_4 \cdot 4H_2O$	22.3	
KI	0.83	0.75
H_3BO_3	6.2	3
$ZnSO_4 \cdot 7H_2O$	8.6	2
$CuSO_4$		0.025
$CuSO_4 \cdot 5H_2O$	0.025	
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25
$CoCl_2 \cdot 6H_2O$	0.025	0.025
$FeSO_4 \cdot 7H_2O$	27.8	
Na_2-EDTA	37.3	
NaFe-EDTA·$3H_2O$		40
ミオ-イノシトール	100	100
チアミン-HCl	0.1	10
ニコチン酸	0.5	1
ピリドキシン-HCl	0.5	1
グリシン	2	

[0044]By the culture medium for fluid shaking passages, the capitulum lump which performed culture for 20 days was dried, and the Soxhlet extraction was performed by MeOH (2.5 to 3 hours). Hardened the extract by drying, it was made to hold to Nihon Millipore Sep-Pak C18 cartridge, and backwashing by water was performed. Fractionation of subsequent MeOH elution was set to Sample of HPLC.

[0045]HPLC analysis was conducted on condition of GURAJUENTO (20mM phosphate buffer solution is included.) of the Fluofix column by NEOSU (diameter xof 4mm150mm), column temperature:40 **, and mobile phase:20% to 80% of CH_3 CN, rate-of-flow:1 ml/min, and detection:UV (203 nm).

[0046]After TLC analysis spotted Sample using product Kieselgel made by Merck 60 F_{254}, it develops by $CHCl_3$:CH_3OH:H_2 O= 65:35:10 (lower layer), and it was heated and was made to color at 120 ** after concentrated-sulfuric-acid spraying. The chikusetsu-saponin group extracted from the plant body rhizome showed coloring of red-purple-blue, and showed a fluorescence under UV.

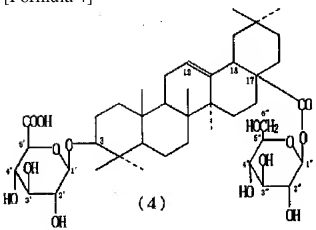
[0047]20 or more peaks were detected in HPLC analysis. When each peak was isolated preparatively by HPLC and TLC analysis was conducted, only two peaks showed purplish red coloring and the fluorescence under UV. Hereafter, about the substance (the high polarity was set to TSB1 and low-polar one was set to TSB2.) of these two peaks, preparative isolation was repeated and isolation refining of the two substances was carried out, respectively (drawing 1).

[0048]When ^{1}H-NMR of TSB2 was measured, the characteristic peak of H and five peaks of sugar were seen the 12 or 18th place, and the integral value showed that it was one glycoside of oleanoric acid of the basic skeleton of oleanoric acid. What it is dramatically similar with the ^{1}H-NMR spectrum of oleanoric acid 3-O-glucoside compounded from oleanoric acid, and the peak of the place [3rd] of H corresponds, It turned out that glucronic acid is ****(ing) at least 6' of glucoside to the OH radical of the 3rd place since the characteristic peak of H is not seen. Although that existence is known by the sugar beet (Suger beet) as for this oleanoric acid 3-O-glucronide, such saponin is not reported from Panax japonicus.

[0049]The elution time of HPLC of the chikusetsu-saponin IVa and mobility of TLC of TSB1 which are detected in very small quantities by the plant body corresponded, and it was suggested that it is the same substance. Coincidence of structure with the chikusetsu-saponin IVa was checked by two-dimensional ^{1}H-NMR analysis (2D-^{1}H-COSY and NOESY) of TSB1.

[0050]

[Formula 4]



[0051]

[Formula 5]